AD

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/00, A61K 39/395, 49/00 A61K 43/00, 47/48, 45/06 G01N 33/577, 33/563

A1

(11) International Publication Number: WO 93/02105

(43) International Publication Date: 4 February 1993 (04.02.93)

(21) International Application Number: PCT/US92/06043

(22) International Filing Date: 17 July 1992 (17.07.92)

(30) Priority data:
732,969 19 July 1991 (19.07.91) US

(71) Applicant: HYBRITECH INCORPORATED [US/US]; 11095 Torreyana Drive, San Diego, CA 92121 (US).

(72) Inventors: GRAUER, Lana, S. ; 13252 Caminito Point, Del Mar, CA 92014 (US). AHLEM, Clarence, N. ; 3976 Sutherland Drive, Palo Alto, CA 94303 (US).

(74) Agents: CAMPBELL, Cathryn et al.; Campbell and Flores, 4370 La Jolla Village Drive, Suite 700, San Diego, CA 92122 (US).

(81) Designated States: AU, CA, JP, KR, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Published
With international search report.

(54) Title: TRIFUNCTIONAL COMPOUNDS HAVING SPECIFICITY FOR MULTI-DRUG RESISTANT CELLS

(57) Abstract

The present invention relates to trifunctional compounds having specific reactivity with P-glycoproteins associated with multi-drug resistant (MDR) tumor cells. The compounds can optionally be specific for diagnostic and/or therapeutic agents as well and are thus contained in pharmaceutical compositions. Methods for detecting and treating patients with tumors expressing P-glycoproteins associated with MDR cells are also provided.

TRIFUNCTIONAL COMPOUNDS HAVING SPECIFICITY FOR MULTI-DRUG RESISTANT CELLS

FIELD OF THE INVENTION

The present invention generally relates to multi-drug resistance exhibited by many tumor cells. More specifically, the present invention relates to trifunctional compounds having specificity for multi-drug resistance (MDR) cells that overexpress P-glycoproteins and to their use for diagnosis and therapy.

BACKGROUND OF THE INVENTION

The ability of certain tumor cells to acquire multi-drug resistance has been a significant barrier to the effective use of chemotherapy in the treatment of such tumors and other neoplastic disorders. Multi-drug resistance is the phenomenon by which certain cancer cells selected for resistance to one drug can simultaneously exhibit resistance to structurally and functionally unrelated drugs to which they have not been previously exposed.

Cells exhibiting the multi-drug resistance phenotype show reduced intracellular accumulation of such drugs compared with the corresponding drug-sensitive cells. A common characteristic of many MDR cells is the overexpression of cell surface P-glycoproteins that are absent or expressed in low levels in drug-sensitive and normal cells. One function of the P-glycoproteins is to act as a drug efflux pump by which intracellular toxins are actively pumped out of the cell. It is believed certain regions of the P-glycoprotein bind to the energy-carrying compound, ATP, which provides the energy to pump the non-related toxins out of the cell.

At least two P-glycoprotein genes have been identified in humans and three in mice, suggesting that a family of such glycoproteins may exist. One human P-glycoprotein has an apparent molecular weight of about 180 Kd, while the other has an apparent molecular weight of about 170 Kd. Monoclonal antibodies specifically reactive with these P-glycoproteins are known and have been described, for example, in Hamada & Tsuruo, PNAS (U.S.A.) 83:7785-7789 (1986) and in European Patent Application No. 87309939.4, published on May 18, 1988. These monoclonal antibodies are commonly referred to as anti-MDR antibodies.

Bispecific anti-MDR antibodies have been reported in van Duk et al., Int. J. Cancer 44:738-743 (1989). These bispecific antibodies have one specificity for the 170 Kd P-glycoprotein and a second specificity for CD3 to induce lysis of MDR tumor cells.

Polyclonal and monoclonal anti-MDR antibodies are limiting since they can only bind to the site of action. If diagnostic or therapeutic agents are also desired, they must be chemically coupled to such antibodies. Bispecific antibody molecules are also limited. For example, such bispecific antibodies have the inherent limitation of effectively being monospecific once bound to the antigen binding site in that they can target only a single agent to such binding site. In addition, such bispecific antibodies are limited in their ability to recognize at most two different antigen binding sites.

It would be advantageous to use compounds that are capable of being more than monospecific at the site of action or have the ability to recognize more than two different P-glycoproteins. For example, one trifunctional compound having three arms each specific

for different P-glycoproteins or different P-glycoprotein binding sites can be used in place of three monoclonal antibodies. Alternatively, a trifunctional compound having one arm specific to a P-glycoprotein, a second arm specific for a diagnostic agent and a third arm specific for a therapeutic agent would be useful to diagnose and treat a patient without administering additional antibodies that would be necessary if monospecific or bispecific antibodies were used.

Thus, a need exists for compounds that have more than two specificities. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to trifunctional compounds of the following Formula:

$$\begin{array}{ccccc} X_1 & \text{———} & L & \text{———} & X_3 \\ & & | & & \\ & & X_2 & & \end{array} \qquad \text{(I)}$$

wherein L is a cross-linking agent, X_1, X_2 and X_3 are antibody Fab′-like moieties, and at least one of the Fab′-like moieties has specific reactivity for a P-glycoprotein associated with a MDR cell. The Fab′-like moieties can also be specific for diagnostic and therapeutic agents. The present invention also provide pharmaceutical compositions containing the trifunctional compounds and pharmaceutically acceptable carriers.

The trifunctional compounds can have arms that recognize up to three different P-glycoproteins or three different antigenic binding sites on the same P-glycoprotein. Alternatively, such compounds can have one

arm specific for a P-glycoprotein and two arms specific for one or two diagnostic agents or therapeutic agents. A particularly useful compound has one arm specific for a P-glycoprotein, a second arm specific for a diagnostic agent and a third arm specific for a therapeutic agent. The desired trifunctional configuration will depend on its intended use.

Particularly useful diagnostic agents are complexes of chelating agents and an imaging agent, such as a radionuclide or a paramagnetic metal ion. Therapeutic agents include cytotoxic agents and complexes of chelating agents and radionuclides.

Also provided are methods for inhibiting the activity of P-glycoproteins in conferring multi-drug resistance to certain tumor cells. Such methods include administering the trifunctional compounds of the present invention. Chemosensitizers can optionally be administered with the trifunctional compounds.

The present invention further provides for the detection of MDR cells. The detection of such cells can be accomplished by in vitro assay of samples or in vivo imaging of a patient. In the in vivo imaging methods, the trifunctional compounds of the present invention are administered either simultaneously with or prior to the administration of a diagnostic agent.

In another aspect, the present invention also provides methods for treating a patient having a disorder associated with MDR cells. The trifunctional compounds of the invention can also be similarly administered either simultaneously with or prior to the administration of a therapeutic agent.

The present invention further relates to

methods for the diagnosis and treatment of a patient having or suspected of having MDR cells by administering a trifunctional compound having specificities for a diagnostic agent and a therapeutic agent.

Finally, kits useful for performing the methods of the present invention are also provided. The kits contain the trifunctional compounds of the present invention and any desired ancillary reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the relative activities of intact LS2H241 antibody, bifunctional F(ab)'2 and trifuncitonal F(ab)'3.

Figure 2 shows the binding of LS2H241 and trifunctional (LS2H)2/xCHA to CEM membranes.

Figure 3 shows the effect of LS2H241 and trifunctional (LS2H)2/xCHA in modulating actinomycin D uptake in multi-drug resistant cells.

Figure 4 shows the results of (LS2H)2/xCHA biodistribution studies in nude mice bearing BROtMDR1 xenographs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to trifunctional compounds having at least one specificity for an antigenic determinant on a P-glycoprotein expressed by multi-drug resistant cells of certain tumors. Such tumors include, for example, ovarian, colon, renal and breast carcinomas, leukemia, bladder carcinoma, lymphoma, myeloma, adrenal carcinoma,

teratocarcinoma, sarcoma, seminoma and the like. Since the various P-glycoproteins are not tissue specific, the compounds of the present invention are likewise not tissue specific. Thus, the compounds have applicability in the management of a broad spectrum of tumors.

The trifunctional compounds of the present invention have the following general Formula I:

$$\begin{array}{ccc} X_1 \text{———} & L & \text{———} X_3 \\ & | & \\ & X_2 & \end{array} \qquad (I)$$

L is a cross linking agent. X_1, X_2 and X_3 can be antibody fragments or antibody-like fragments, particularly Fab′-like moieties, having a combination of various antigenic specificities. As used herein, the term "Fab′-like moiety," when referring to X_1, X_2 or X_3, is used interchangeably with the term "arm." The term "specificity" refers to the specific binding reactivity of a compound with a particular antigenic determinant.

The term "trifunctional," as used herein, refers to the three functional arms or valencies of a compound. Thus, a trifunctional compound can also be referred to as a "trivalent" compound.

A trifunctional compound can be monospecific, bispecific or trispecific. Used in this sense, the term "specific" refers to the number of different antigenic determinants or epitopes a particular compound can bind. For example, a bispecific, trifunctional compound has one arm specific for an antigenic determinant, while the other two arms are specific for a different antigenic determinant. The arms of a trispecific, trifunctional compound each have specificities for different antigenic determinants relative to the other two arms, i.e., three

unique determinants. The antigenic determinants recognized by the bispecific and trispecific compounds of the present invention can be from the same antigen or different antigens. The arms of a monospecific, trifunctional compound are specifically reactive with the same antigenic determinant.

The term "Fab′-like moiety," also referred to herein as an "Fab′-like fragment," includes Fab or longer Fab′ fragment of an antibody, as described in the U.S. Patent Application Serial No. 07/491,406, filed on March 9, 1990 (Attorney Docket No. H-7912), by Ahlem and Huang, which is incorporated herein by reference. As is known to those skilled in the art, a Fab′ fragment includes a portion of the constant Fc region that is not included in a Fab fragment. The Fab and Fab′ fragments include the Fv fragment, which is the variable region of an antibody that determines the antigen binding specificity. The Fab′-like fragment can be obtained directly from an antibody by digesting the antibody into the desired fragments or indirectly by chemical synthesis of a polypeptide having the amino acid sequence of a Fab or Fab′ fragment from such an antibody. The Fab′-like fragment can be a single chain antibody derived from the binding region of an antibody as described in U.S. Patent No. 4,946,778, issued August 7, 1990 to Ladner et al., which is incorporated herein by reference. Alternatively, the fragments can be obtained indirectly from an antibody by recombinant engineering techniques well known in the art.

The Fab′-like moieties can also be obtained from chimeric antibodies, particularly those having a human constant region (Fc) and a non-human variable region (Fv), such as a murine Fv region. The human constant region minimizes the risk of inducing an immune response when the compounds of the present invention

having a variable region from a non-human species are administered to a human. For example, the potential immunogenicity of murine antibodies in humans, referred to as HAMA (human anti-murine antibody), can result in severe adverse reactions with repeated administration of murine antibodies. Those skilled in the art will appreciate that desirable compounds can be selected for non-human mammals based on similar considerations for specie differences.

As used herein, "mammal" is used interchangeably with the term "patient" and means any mammal capable of expressing a P-glycoprotein associated with multi-drug resistant cells, including, for example, dogs mice, hamsters, humans, and the like.

The trifunctional compounds of the present invention have at least one arm directly or indirectly derived from an antibody having specificity for a P-glycoprotein associated with MDR cells. Such antibodies are generally referred to as anti-MDR antibodies. Anti-MDR antibodies from which the trifunctional compounds are derived can be polyclonal, monoclonal, chemically synthesized or recombinantly produced. Anti-MDR antibodies are known in the art and include, for example, MRK16 and MRK17 antibodies described in Hamada & Tsuruo, PNAS (U.S.A.) 83:7785-7789 (1986) and C219 described in U.S. Patent No. 4,837,309, issued June 6, 1989 to Ling et al. The antibodies listed in Table 1 can also be used, particularly the monoclonal antibody produced by the hybridoma designated as LS2H241 ATCC DEPOSIT NO.: HB 10814. The antibodies were generated following the procedure of Example I.

Table 1

Drug Resistance Related Monoclonal Antibodies

ELISA Ratios[a]

Antibody	Isotype	MC-IXC/VCR / MC-IXC	SH-SY5Y/VCR / SH-SY5Y	P-glycoprotein[b]
HYB-241	IgG_1	40	12	+
HYB-612	IgG_1	17	19	+
HYB-034	IgG_1	27	13	+
HYB-057	IgG_1	7	11	+
HYB-237	IgG_1	97	6	+
HYB-374	IgG_1	7	11	+
HYB-162	IgG_{2b}	41	3	+
HYB-243	M	50	8	+
HYB-195	M	1	5	-
HYB-065	M	55	1	-

[a]Antibody binding ratios, resistant sensitive

[b]Antibody reacts with the human MDRI transfectant cell line, BROtMDRI and not the parental nontransfected cell line by ELISA

The anti-MDR antibodies recognize epitopes on different P-glycoproteins having molecular weights in the range of about 170-180 kilodaltons (Kd). The existence of a family of MDR-associated P-glycoproteins has been reported in Meyers et al., "Characterization of Monoclonal Antibodies Recognizing a 180 Kd P-Glycoprotein: Differential Expression of the 180 kDa and 170 kDa P-Glycoproteins in Multidrug-resistant Human

Tumor Cells," Cancer Res. 49:3209-3214 (1989). For example, antibody LS2H241 recognizes an epitope on a P-glycoprotein having an apparent molecular weight of about 180 Kd. Antibody C219 recognizes an epitope on a P-glycoprotein having an apparent molecular weight of about 170 Kd.

One desired function of the anti-MDR Fab′-like moieties is to target the trifunctional compound to cells exhibiting the MDR phenotype for diagnostic or therapeutic purposes. Another function of the anti-MDR Fab′-like moiety is to inhibit or block the P-glycoprotein drug efflux pump activity in conjunction with the administration of MDR drugs in order to facilitate the intracellular accumulation of such drugs and consequent cellular death.

The trifunctional compounds of the present invention can also have Fab′-like moieties directed to antigens, haptens or epitopes (collectively referred to as "antigens") other than the MDR-associated P-glycoproteins. For example, such antigens can optionally be diagnostic agents for the in vitro detection of P-glycoproteins or in vivo imaging of tumors expressing such P-glycoproteins. The antigens can optionally be therapeutic agents for the treatment of a patient having a disorder, such as cancer or other neoplastic disorders, associated with MDR cells.

The term "diagnostic agent" generally means any agent capable of being detected either with or without the aid of instrumentation. When used for in vivo detection, a diagnostic agent is also referred to herein as an imaging agent. A detectable marker can be directly or indirectly attached to a diagnostic agent. Useful diagnostic agents are described U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, by Ahlem and

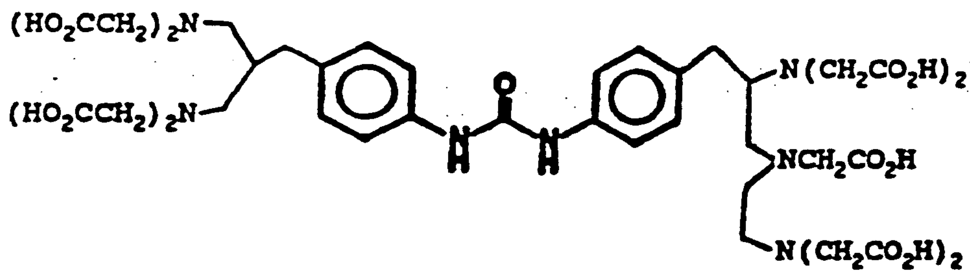
Huang, which is incorporated herein by reference. Such agents include, for example, enzyme detection systems, radionuclides or paramagnetic metal ions. Conventional diagnostic agents for the _in vitro_ detection of target antigens can also be used in the present invention and are well known to those skilled in the art.

Imaging agents useful in the present invention permit the imaging of a patient's organ or tissue for the _in vivo_ detection of tumors expressing P-glycoproteins. The _in vitro_ or _in vivo_ detection of MDR cells and tumors aid in the diagnosis of a disorder or disease associated with the overexpression of such P-glycoproteins. Although the imaging agent can be a radionuclide or paramagnetic metal ion attached directly to an antigen recognized by an arm of a trifunctional compound, preferably the imaging agent is a physiologically compatible chelate complex that has been formed between either a chelating agent and a radionuclide or a chelating agent and a paramagnetic metal ion. For example, radionuclides permit the imaging of tissue and/or organs by gamma scintillation photometry, positron emission tomography (PET), single photon emission computed tomography (SPECT) and gamma camera whole body imaging, while paramagnetic metal ions permit visualization by magnetic resonance imaging (MRI). Such imaging techniques are well known to those skilled in the art.

The term "physiologically compatible chelate complex," as used herein, means a complex between a chelating agent and a radionuclide or paramagnetic metal ion that does not permit physiologically incompatible amounts of the radionuclide or ion to dissociate from the complex _in vivo_.

Suitable complexes and chelating agents are

identified in U.S. Patent Application Serial No. 07/491,406, filed on March 9, 1990 and incorporated herein by reference. Methods for preparing bifunctional derivatives of chelating agents are described in Meares et al., U.S. Patent No. 4,678,667, issued July 7, 1987, incorporated herein by reference, and in Moi et al., J. Am. Chem. Soc., 110:6266 (1988). The chelating agents described therein form physiologically compatible chelate complexes with a variety of metal ions. Other suitable chelating agents are disclosed in Gries et al., U.S. Patent No. 4,647,447, which is incorporated herein by reference. A particularly useful chelating agent is DBX, the use of which is described in more detail in the examples herein. DBX has the following chemical structure:



(II)

DBX is prepared by the reaction of the In3+ complex of (p-aminobenzyl)EDTA and (p-aminobenzyl)DTPA with disuccinimidyl carbonate. To synthesize DBX, the indium complex of (p-aminobenzyl)EDTA and (p-aminobenzyl)DTPA are reacted with disuccinimidyl carbonate in an aqueous solution. Reaction of the aromatic amine groups of (p-aminobenzyl)EDTA and (p-aminobenzyl)DTPA with the disuccinimidyl carbonate yields DBX, which contains a urea linkage between the benzyl DTPA and In-benzyl EDTA moieties. The crude DBX reaction mixture is purified by liquid chromatography using a C18 reversed phase HPLC column, followed by anion exchange on a Sephadex DEAE column (Pharmacia, Piscataway, N.J.). Sephadex is a register trademark of Pharmacia. Other methods of preparing DBX can be readily determined by

those skilled in the art.

Radionuclides that are suitable as imaging agents include gamma-emitting radionuclides, for example, ^{113m}In, ^{67}Ga, ^{99m}Tc, ^{51}Cr, ^{197}Hg, ^{203}Hg, ^{169}Yb, ^{85}Sr, ^{87}Sr, and particularly ^{111}In. These gamma-emitting radionuclides are chelatable in ionic form. The preparation of chelated radionuclides that are suitable for binding by Fab′ fragments is taught by Nicoletti et al, U.S. Patent No. 4,659,839, which is incorporated herein by reference.

Paramagnetic metal ions that are suitable for use as imaging agents in MRI include, for example, the lanthanide elements of atomic number 57-70, or the transition metals of atomic numbers 21-29, 42 or 44. Gries et al. in U.S. Patent No. 4,647,447, incorporated herein by reference, describe the use of chelated paramagnetic metal ions for imaging.

One or two arms of the trifunctional compounds of the present invention can have specificity for a therapeutic agent. A "therapeutic agent," as used herein, refers to any agent administered to a patient to treat a disease, condition or other disorder associated with MDR cells, such as tumor cells that overexpress a cell surface P-glycoprotein.

The trifunctional compounds of the present invention can have direct or indirect specificity for a therapeutic agent. Direct specificity means a functional arm is specifically reactive with the therapeutic agent. Indirect specificity, for example, means a functional arm is specific for a substrate conjugated to a therapeutic agent. Thus, for an enzyme substrate, an enzyme or active fragment thereof is required to activate the therapeutic agent by releasing it from the substrate. More details of the direct and indirect specificities are

provided in U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, which is incorporated herein by reference.

Suitable therapeutic agents of the present invention can be cytotoxic agents that are useful in the treatment of neoplasms. Such cytotoxic agents or drugs include, for example, the vinca alkaloids, the daunomycin family, the mitomycins, the bleomycins and colchicines. Drugs associated with MDR are particularly useful and are referred to herein as MDR drugs. Such drugs include, for instance, doxorubicin, daunorubicin, mitomycin C, etoposide, vinblastine, vincristine, colchicine, dihydroxyanthracenedione, mitoxanthrone, mithramycin, actinomycin D, puromycin, gramicidin D, ethidium bromide, taxol, tenoposide, cytochalasin B, 1-dehydrotoastosterone and the like.

Alternatively, such therapeutic agents can be chelate complexes that are formed between chelating agents and radionuclides, for example, ß-emitters that emit ß- particles of sufficient energy and frequency so as to provide a beneficial effect in the treatment of such disorders. Suitable ß-emitters include ^{67}Cu, ^{186}Rh, ^{188}Rh, ^{189}Rh, ^{153}Sm, ^{111}In (AÜger), and particularly ^{90}Y. The ß-emitters are chelatable when in ionic form, typically when in the +1 to +4 oxidation state. Chelating agents useful for such complexes are described in U.S. Patent Application Serial No. 07/491,406, and in Meares et al., U.S. Patent No. 4,678,667, issued July 7, 1987, both of which are incorporated herein by reference, and in Moi et al., J. Am. Chem. Soc., 110:6266 (1988).

Suitable cross linking agents are also described in U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, which is incorporated herein by reference. Particularly useful cross linking

agents for the trifunctional compounds of the present invention are the tris-maleimides described in U.S. Patent Application Serial No. 07/491,386, filed March 9, 1990, which is incorporated herein by reference.

Methods for preparing trifunctional compounds are described in U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, the methods of which are incorporated herein by reference. Other methods of preparing such compounds are described in the examples herein.

The trifunctional compounds of the present invention can be used for a variety of purposes, including use in the diagnostic and therapeutic methods of the present invention. The compounds can also be used to purify MDR-associated P-glycoproteins. For example, if a mixture of different P-glycoproteins is desired, the trifunctional antibodies would be particularly useful to isolate such proteins from a sample. Those skilled in the art can readily identify other potential uses for the trifunctional compounds.

The present invention further provides pharmaceutical compositions comprising a trifunctional compound of Formula I and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers are well known and include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline supplemented with 5% dextrose or human serum albumin, if desired.

The pharmaceutical compositions, useful for imaging and therapy, can be prepared by methods known in the art, including the simple mixing of the reagents. Those skilled in the art will know that the choice of pharmaceutical carrier and the appropriate preparation of

the composition will depend on the intended use and mode of administration.

The present invention also provides methods of inhibiting the ability of the P-glycoprotein molecule to pump drugs out of the cell across the membrane. The methods involve contacting an MDR cell with a trifunctional compound of Formula I. The trifunctional compound can be monospecific, bispecific or trispecific to different epitopes or different P-glycoproteins. Trispecific, trifunctional compounds are particularly useful over known monospecific and bispecific antibodies since more potential binding sites can be blocked with one compound compared with a monospecific or bispecific antibody. Although the exact mechanism is not known, it is believed that the binding of the LS2H241 compound at the cell surface may induce a conformational change in the P-glycoprotein molecule that interferes with the ability of the molecule to pump drug out of the cell across the membrane. These methods can therefore be used for therapeutic purposes to enhance the intracellular accumulation of MDR drugs.

The methods further include contacting the cells with a chemosensitizer, such as verapamil, either prior to, simultaneously with or subsequent to contacting such cells with the trifunctional compound. Chemosensitizers are compounds that compete with MDR drugs as substrates for binding to the ATP dependent efflux pump, thus rendering multi-drug resistant tumor cells sensitive to drugs that would otherwise be ineffective. Such chemosensitizers or modulators include calcium channel blockers as described in Tsuruo et al., Cancer Res. 41:1967-1972 (1981); calmodulin inhibitors as described in Ganapathi & Grabowski, Cancer Res. 43:3696-3699 (1983); antiarrythmics as described in Tsuruo et al., Cancer Res. 44:4403-4407 (1984) and Chauffert et

al., _Br. J. Cancer_ 56:199-222 (1987); antimalarials as described in Zamora & Beck, _Biochem. Pharm._ 35:4303-4407 (1986); other lysoosmotropic agents as described in Zamora et al., _Mol Pharmacol._ 33:454-462 (1988); steroids as described in Yan et al., _J. Biol. Chem._ 264:782-788 (1989); antiestrogens as described in Ramu et al., _Cancer Res._ 44:4392-4395 (1984); and cyclic peptide antibiotics as described in Slater et al., _J. Clin. Invest._ 77:1405-1408 (1986). However, many chemosensitizers, such as verapamil, are highly toxic. The use of the trifunctional compounds allows the use of a less toxic dose of such chemosensitizers to obtain similar results and thus reduce the toxic side effects.

As used herein, the term "contacting" encompasses any means by which two substances come into close physical proximity. For example, the trifunctional compound comes into contact with a P-glycoprotein when it comes into physical proximity sufficiently close for the compound to bind to an antigenic determinant on the glycoprotein. For the _in vitro_ methods of the present invention, such contact can be accomplished, for example, by adding the compound to a sample or dipping the sample into a solution containing the compound. For the _in vivo_ methods, such contact can be accomplished by administering the compound to a patient.

As used in the methods of the present invention, the trifunctional compounds can be administered to a mammal by any means known to those skilled in the art, including parenteral injection or topical application. Injection can be done intravascularly, intraperitoneally, subcutaneously or intramuscularly. For parenteral administration, the compounds can be administered in admixture with a suitable pharmaceutically acceptable carrier.

The dosage regimen for diagnosing and treating MDR-associated disorders depends on a variety of factors, including the type of disorder, age, weight, sex and medical condition of the patient, the severity of the condition, the route of administration and the type of diagnostic or therapeutic agent used. A skilled physician or veterinarian can readily determine and prescribe the effective amount of the compound or pharmaceutical composition required to diagnose or treat the patient. Conventionally, one skilled in the art would employ relatively low doses initially and subsequently increase the dose until a maximum response is obtained. Because the trifunctional compound of the present invention localizes the diagnostic or therapeutic agent, much lower doses are believed to be effective than with conventional methods.

Methods for detecting the presence or absence of a multi-drug resistant cell are also provided by the present invention. Such methods include (a) contacting a sample containing or suspected of containing a multi-drug resistant cell with a detectable trifunctional compound of Formula I, said trifunctional compound having at least one Fab′-like moiety specific for a diagnostic agent; and (b) detecting the binding of the compound to the sample, whereby binding indicates the presence of a multi-drug resistant cell in the sample. In these methods, at least one of the Fab′-like moieties of the trifunctional antibody is specific for a diagnostic agent and another is specific for a P-glycoprotein associated with a MDR cell.

The trifunctional compound can have any combination of specificities described previously. Particularly useful compounds have one specificity for a diagnostic agent and the remaining two arms are specific for two different epitopes of the same P-glycoprotein, or preferably, different P-glycoproteins. Thus, the

trifunctional compound of the present invention is particularly useful for assaying samples that contain a mixture of P-glycoproteins.

Assays useful in the in vitro methods include any immunoassay known to those skilled in the art in which antibody binding is determinable. Such immunoassays include, ELISA, agglutination assays, flow cytometry, serum diagnostic assays, immunohistochemical assays, including immunoperoxidase staining of tissue sections and others described, for example, in R. Aloisi, Principles of Immunology and Immunodiagnostics, 152-176 and 221-222 (1988), which is incorporated herein by reference.

In a further aspect, the present invention relates to methods for detecting tumors expressing P-glycoproteins in a mammal having or suspected of having such tumors. The methods include (a) administering the trifunctional compound of Formula I, wherein at least one of the Fab-like moieties has specific reactivity with a diagnostic agent; (b) administering a diagnostically effective amount of the diagnostic agent; and (c) detecting the binding of the trifunctional compound to the mammal, whereby binding indicates the presence of a tumor expressing P-glycoprotein. The methods can be used to diagnose or monitor a patient or to determine the appropriate therapy. Such determinations depend on the extent of binding. For example, a diagnosis can be made by those skilled in the art by comparing the extent of binding in a patient or patient sample with the extent of binding in a control, such as a mammal free of the disorder. Thus, an elevated amount of binding compared to such a control can be diagnostic of the disorder.

For the in vivo detection methods, the trifunctional compounds have one arm specific for a P-

glycoprotein associated with a MDR cell and a second arm specific for an imaging agent. Suitable imaging agents have been identified above. The third specificity can be against the same or different P-glycoprotein, or against the same or different imaging agent. For monitoring, the third specificity is preferably a different imaging agent.

The trifunctional compound and the imaging agent can be administered to the patient simultaneously or sequentially. For sequential administration, the imaging agent can be administered after the trifunctional compound is given sufficient time to localize to the P-glycoprotein sites, preferably about 24 hours. One skilled in the art can readily determine the appropriate time period.

The present invention further relates to methods for treating a mammal having a disorder associated with multi-drug resistant cells. These methods involve (a) administering a therapeutically effective amount of a trifunctional compound of Formula I, wherein at least one of said Fab′-like moieties has specific reactivity with a therapeutic agent; and (b) administering a therapeutically effective amount of the therapeutic agent to the mammal. Suitable therapeutic agents have been identified above.

The trifunctional compounds of the therapeutic methods can have any combination of specificities as long as one specificity is against a MDR-associated P-glycoprotein and a second specificity is against a therapeutic agent. The third specificity can be against either the same or a different P-glycoprotein associated with MDR or against the same or different therapeutic agent.

The methods herein advantageously provide simultaneous inhibition of P-glycoprotein activity and delivery or focusing of therapeutic agents to the site in order to kill the MDR cells. Similar to the in vivo detection methods, the trifunctional compound and therapeutic agent can be administered simultaneously or sequentially.

Methods for diagnosing and treating a mammal having or suspected of having a disorder associated with multi-drug resistance are advantageously provided by the present invention. Such methods include (a) administering a trifunctional compound of Formula I to the mammal, wherein one Fab′-like moiety has specific reactivity with a P-glycoprotein, a second Fab′-like moiety has specific reactivity with a diagnostic agent and a third Fab′-like moiety has specific reactivity with a therapeutic agent; (b) administering a diagnostically effective amount of the diagnostic agent to the mammal; (c) detecting the binding of the trifunctional compound to the mammal, wherein an elevated amount of binding of the compound is diagnostic of the disorder; and (d) if warranted, administering a therapeutically effective amount of the therapeutic agent to the mammal. Because these methods use one trifunctional compound to diagnose and, if needed, treat a patient, the risk of HAMA is significantly reduced.

Finally, kits useful for performing the methods of the present invention are also provided. The kits contain a trifunctional compound of the present invention and any necessary or desired ancillary reagent. Such ancillary reagents can be diagnostic or therapeutic agents, signal detection systems, buffers, stabilizers, pharmaceutically acceptable carriers and the like.

The following examples are intended to

illustrate, but not limit, the present invention.

EXAMPLE I

Production of MDR Antibodies

Monoclonal antibodies were produced either against the multi-drug resistant human neuroepithelioma cell line MC-IXC/VCR or the multi-drug resistant human neuroblastoma cell line SH-SY5Y/VCR. These cell lines were provided by Dr. June Beidler (Sloan-Kettering Cancer Center, New York) and are described in Scotto et al., Science 232:751-755 (1986).

To produce the LS2H241 hybridoma, balb/c mice were immunized with 5 x 10^6 SH-SY5Y/VCR cells intraperitoneally. Three weeks later, the mice received a second intraperitoneal injection of 5 x 10^6 of the same cells. Four days prior to fusion, the mice received a final intravenous boost of 5 x 10^6 cells.

Cell fusion was carried out according to the procedure of Kohler and Milstein, Nature 256:495-497 (1975) as modified by Gerhard, Monoclonal Antibodies, R. Kennett et al., 370-371 (Plenum Press, 1980). Briefly, 108 splenocytes were fused with 2.5 x 10^7 P3-X63-Ag8.653 (ATCC #CRL 1580), a mouse myeloma cell line, in 1.0 ml of 35% polyethylene glycol (PEG 1500) in AP-MEM medium. Following the fusion, the cells were cultured in medium supplemented with hypoxanthine, aminopterin and thymidine at 370C in a humidified 5% CO_2 incubator.

Initial screening of hybridoma cultures was done by indirect enzyme-linked immunoabsorbent assay (ELISA) using a horseradish peroxidase conjugate of sheep anti-mouse Ig (Cappel, Malvern, Pennsylvania). Drug-sensitive and drug-resistant neuroblastoma cell lines, which grow as monolayers, were placed in microtiter

plates and allowed to grow overnight. Cells growing in suspension were added to microtiter plates that had been coated with 0.1% poly-L-lysine according to the procedure of Cobbold, J. Immuno. Methods 44:125-133 (1981).

The following day, cells were fixed with 0.125% glutaraldehyde. Following removal of the fixative and blocking with 10% bovine serum albumin, 40 μl of hybridoma supernatant was added and allowed to incubate for one hour at 37°C. Plates were washed with PBS and incubated with peroxidase-conjugated sheep anti-mouse immunoglobulin (Ig). After five washes with PBS, positive clones were identified by addition of 100 μl of a solution containing 1 mg/ml o-phenylenediamine, 0.1% hydrogen peroxide, 50 mM citrate and 100 mM sodium phosphate buffer (pH 5.0). The plates were read at 490 nm on an EIA Autoreader (Bio-Tek Instruments, Winooski, Vermont).

Ratios were determined by dividing the signal obtained on the drug-resistant lines by the signals obtained on the corresponding drug-sensitive lines. Hybridomas that demonstrated a 6-fold or greater reactivity with the resistant cell lines relative to the drug-sensitive cell lines were screened again in the same manner. Antibodies that continued to demonstrate a 5-fold or greater reactivity were selected for further characterization.

The other MDR antibodies were generated following the same general procedure. Two fusions produced 1,139 hybridomas, of which fifteen clones consistently demonstrated greater reactivity with drug-resistant cells relative to the corresponding drug-sensitive cells. These clones are identified in Rittmann-Grauer et al., "Monoclonal Antibodies To Target Multidrug-Resistant Cells," AACR Proceedings 20:394

(1987). The hybridoma producing monoclonal antibody LS2H241 (also referred to as HYB 241) ATCC No. HB 10814 was selected for synthesizing a trifunctional compound of the present invention.

EXAMPLE II

Synthesis of LS2H $F(ab)'_2$ Fragments

$F(ab)'_2$ fragments of monoclonal antibody LS2H241 were prepared according to the procedures of Rousseaux *et al.*, *J. Immunol. Methods* 64:141-146 (1983), which is incorporated herein by reference. Thirty-three ml of LS2H241 monoclonal antibody at 5.9 mg/ml was dialyzed in 2 L of a 0.1 M sodium formate and 0.1 M NaCl solution (pH 2.8) overnight at 4°C. The dialysis buffer was changed to 0.1 M NaOAc and 0.1 M NaCl (pH 4.5). The LS2H241 antibody was again dialyzed overnight at 4°C. The antibody solution was digested with 3% pepsin (5.7 mg) at 37°C for 110 minutes. The digestion was then terminated with 1 ml 2M tris-Cl (pH 8.0) and 100 μl 10 M NaOH with a final pH of about 8.0. The terminated digest was then dialyzed in 1 L borate buffered solution (BBS: 50 mM sodium borate, 50 mM NaCl, pH 8.2) for 48 hours at 4°C.

EXAMPLE III

Synthesis of xCHA $F(ab)'_2$ Fragments

The antibody designated herein as "xCHA" is a mouse human chimeric antibody having specificity for the In-EDTA chelate complex. The xCHA antibody was prepared by essentially the same method used for the preparation of chimeric CEM antibody as described in U.S. Patent Application Serial No. 07/274,106, by M.J. Johnson, filed on November 17, 1988, incorporated herein by reference. The monoclonal antibody from which the murine variable region of xCHA was derived is described in U.S. Patent

No. 4,722,892, issued to Meares et al., incorporated herein by reference.

The preparation of xCHA F(ab)′ fragments is described in U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, incorporated herein by reference. Briefly, xCHA antibody solution having a concentration of 5-15mg/ml were dialyzed overnight in an acetate buffered saline solution (pH 4.1) overnight at 4°C. A concentrated pepsin solution (2% of the antibody mass) was added to the dialyzed solution and incubated for about 4-24 hours at 37°C. The reaction was terminated by adding 50-100 mM tris buffer and adjusting the pH to about 8 with 10 N NaOH. The terminated digests were dialyzed against BBS (pH 8.2). The $F(ab)'_2$ fragments were purified by either HPLC or gel filtration on a Sephadex G-150 column (Pharmacia, Piscataway, N.J.).

EXAMPLE IV

Synthesis of Trifunctional Compound LS2H(2)/xCHA

A trifunctional compound of Formula I was synthesized using the $F(ab)'_2$ fragments of Examples II and III, in which elements X_1 and X_2 were derived from the LS2H241 fragments and X_3 was derived from the xCHA fragment. The synthesis of the trifunctional compound was generally in accordance with the procedures set forth in U.S. Patent Application Serial No. 07/491,406, filed March 9, 1990, incorporated herein by reference.

First, 66 ml of a solution of the LS2H241 $F(ab)'_2$ fragment (4.5 mg/ml) in BBS was incubated with 14 µl 0.5 M DTPA at 37 °C for 15 minutes. Then, 280 µl of 0.5 M cysteine in a citrate-DTPA buffer (50 mM ammonium citrate, 0.1 M NaCl and 0.5 mM diethylenetriaminepentaacetic acid (DTPA) solution; pH 6.3) were added to the antibody fragment mixture and

incubated for an additional 10 minutes at 37°C.

After reduction, excess cysteine was removed by gel filtration on a 90 ml column of P-6 DG (BioRad Laboratories, Richmond, California) in citrate-DTPA buffer. The protein fraction was collected by monitoring absorbance at 280 nm. The volume of the protein fraction (Fab', molecular weight 50 Kd) was 11 ml, the absorbance at A280 was 2.97, which is equivalent to a protein concentration of 2.1 mg/ml. The reactive sulfhydryl content of the Fab' product was determined with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) in BBS (pH 8.2). The absorbance was measured at 412 nm using a molar extinction coefficient of 13,600. The absorbance at 412 nm was 1.8, which is equivalent to 132 μM sulfylhydryl. The sulfhydryl to Fab' ratio was determined to be 3.2.

To prepare the xCHA fragment, 1.4 ml of xCHA F(ab)'$_2$ was incubated with 3 μl of 0.5 M DTPA for 15 minutes at 37°C, followed by the addition of 2.8 μl of 0.5 M dithiothreitol (DTT). The resulting solution was incubated for an additional 10 minutes at 37°C. Excess DTT was removed on a 15 ml (1.5 x 28 cm) P-6 DG column in citrate-DTPA buffer. The Fab' fraction was collected by monitoring at 280 nm. The total volume collected was 4.4 ml. The absorbance at A280 was 3.07, which is equivalent to 2.2 mg/ml. Free sulfhydryl concentration was 180 μM as determined with DTNB as described above. The sulfhydryl to Fab' ratio was 4.1.

The LS2H Fab' fraction was derivatized with a 30 fold molar excess of N,N'-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine (BMP), in which 5.4 mg BMP were dissolved in 400 μl of DMF/water (50:50) followed by the addition of 10.8 ml of the LS2H Fab' fraction with stirring. The derivatization was allowed to proceed at room temperature for 10 minutes. Excess BMP was removed

on a 240 ml P-6 DG column in citrate-DTPA buffer. The Fab'-BMP fraction was collected by monitoring at an absorbance of A280 nm. The total volume collected was 16.1 ml, with an absorbance of 1.82, which is equivalent to 1.3 mg/ml (26 μM). The active maleimides, as determined by cysteine back titration, were present at a concentration of 22 μM, which represents an average of 0.85 maleimides/Fab'.

A 15.5 ml (20 mg) sample of the LS2H Fab'/BMP solution was added with stirring to 3.5 ml (7.7 mg) of the xCHA Fab' solution. The reaction was incubated at room temperature for 1 hour and terminated by the addition of 20 μl of 1 M NEM in DMF.

The Fab'_3 trifunctional and Fab'_2 bifunctional products were purified on a 2.5 x 100 cm G-150 SF column in BBS (pH 8.2). Column fractions were analyzed by gel filtration HPLC and SDS-PAGE. Fractions 39-43, corresponding to the 150 Kd product, LS2H(2)/xCHA, were pooled. The volume was 22.5 ml, with an absorbance at A280 of .469, which is equivalent to 0.33 mg/ml. The Fab'3 trifunctional product (7.4 total mg) was recovered after purification.

The Fab'3 trifunctional compound contained two LS2H241 Fab' fragments and one xCHA Fab' fragment. The bifunctional Fab'2 compound contained one LS2H241 Fab' fragment and one xCHA Fab' fragment.

EXAMPLE V
In Vitro Studies

A. Comparison of Activity

The activities of Fab'_3, Fab'_2 and intact monoclonal antibody LS2H241 were compared by ELISA to

determine if the preparation of the bifunctional and trifunctional compounds resulted in any loss of activity. The antibody compounds were tested against the CCRF-CEM cell line, which is drug-sensitive, and CEM/VLB_{100}, the corresponding drug-resistant cell line. Both cell lines were obtained from William Beck, et al., Cancer Res. 39:2070-2076 (1979).

To perform the membrane ELISA, 5 μg of sucrose purified CCRF-CEM membranes and CEM/VLB_{100} membranes were aliquoted into wells of flat bottom microtiter plates (Dynatech, Alexandria, Virginia). The plates were incubated at 37°C in an oven overnight. The plates were washed three times with cold tap water and then incubated for 30 minutes at room temperature with 4% bovine serum albumin in phosphate-buffered saline. The buffer was removed and 50 μl of one of the antibodies (intact, Fab'_2 or Fab'_3) was added to the wells and incubated at room temperature for one hour. Five concentrations of antibody were assayed (1 ng/ml, 10 ng/ml, 100 ng/ml, 1 μg/ml and 10 μg/ml). The plates were washed six times with tap water prior to the addition of peroxidase conjugated goat anti-mouse kappa and lamda immunoglobulins (Southern Biotechnology, Birmingham, Alabama) at a 1:500 dilution. The plates were incubated for one hour at room temperature and then washed five times with tap water. Color was developed after the addition of 100 μl of 1 mg/ml o-phenylenediamine, 0.03% hydrogen peroxide in 0.1 M citrate phosphate buffer (pH 5.0). The plates were incubated in the dark with shaking for 20 minutes. The reaction was quenched by adding 50 μl of 4 N H_2SO_4 to each well. The wells were then read on an ELISA reader at an absorbance of 490 nm.

As shown in Figure 1, the LS2H241-containing compounds bound to the multi-drug resistant CEM/VLB_{100} membranes, which are known to express P-glycoprotein. By

comparison, as shown in Figure 2, the LS2H241-containing compounds bound minimally to the drug sensitive CCRF-CEM membranes, which are known to contain very low levels of P-glycoprotein. Thus, the ability of the trifunctional compound to bind P-glycoprotein does not appear to be significantly affected by the chemical synthesis of the product.

B. Modulation of P-glycoprotein

To determine the effect of cytotoxic drugs in the presence and absence of intact LS2H241 or Fab'$_3$ trifunctional antibody, MC-IXC/VCR drug-resistant cells were plated out into 2 ml wells at 10^6 cells/well in the absence of vincristine. Cells were incubated overnight and allowed to attach to the wells. The next day, the media was removed and replaced with 1 ml of fresh media containing 50 μg/ml of one of the antibodies. Control wells received fresh media with PBS or a control antibody, GDJ352, which does not bind to P-glycoprotein. Plates were incubated for 30 minutes at 37°C, followed by removal of media and replacement with media containing 50μg/ml of intact or Fab'$_3$ antibody plus ^{3}H-actinomycin D at 0.3 μCi/ml. Control wells were replaced with fresh media with the same amount of ^{3}H-actinomycin D but without Fab'$_3$. The cells were incubated in the presence of the drug for 4 hours at 37°C in 5% CO_2. The supernatants were removed from the wells and the cells were carefully and rapidly washed by serial dipping in 3 beakers of cold PBS. The cells were then released from the wells by mild trypsinization (250 μl of trypsin) and the entire suspension was transferred to a scintillation vial and counted.

The results of the study are shown in Figure 3. Preincubation of the drug-resistant cells with the Fab'$_3$ trifunctional compound prior to incubation with

actinomycin D resulted in an approximately 4 fold increase in intracellular drug concentration over the control cells. Similarly, preincubation of the cells with intact LS2H241 resulted in an approximately 5 fold increase in intracellular drug concentration over the control cells. The data demonstrate that the trivalent compound binds to the drug resistant cell line and blocks the ATP-dependent efflux of drug from the cells.

EXAMPLE VI

Synthesis of the Chelating Agent DBX

The chelating agent, DBX, was prepared by the reaction of disuccinimidyl carbonate with the In^{3+} complex of p-aminobenzylethylene-diaminetetraacetic acid, referred to herein as (p-aminobenzyl)EDTA, and p-aminobenzyldiethylenetriamine-pentaacetic acid, referred to herein as (p-aminobenzyl)DPTA.

A. Synthesis of the In^{3+} complex of (p-aminobenzyl)EDTA

The following reagents were used to prepare the complex: (p-aminobenzyl)EDTA, indium metal, at least 99.99% pure, 6 M Ultrex HCl (J.T. Baker Chemical Co., Phillisberg, New Jersey), distilled water and 10 M NaOH. First 344 mg of indium metal (foil or pellets) were weighed out in an acid-washed 100 mL polypropylene volumetric flask. After 20 ml of 6 M Ultrex HCl was added, the flask was lightly stoppered and placed in a 60°C water bath overnight to hasten dissolution. After the indium was dissolved, the flask was cooled to room temperature, followed by the addition of distilled water to volume. The molar concentration of In^{3+} was calculated as approximately 30 mM using the mass determined in the initial weighing step and 114.82 as the atomic weight for indium.

A 50 mM solution of (p-aminobenzyl)EDTA in distilled water was prepared. The concentration of the solution was determined by measuring the absorbance of a solution at 240 mn and using $\epsilon = 10{,}8000\ M^{-1}\ cm^{-1}$. PBS (10 mM Na_2HPO_4, 150 mM NaCl, pH 7.2) was used as the diluent.

The total moles of (p-aminobenzyl)EDTA was calculated. An aliquot of the indium chloride solution was added to the (p-aminobenzyl)EDTA solution such that the indium was 10% in excess over the chelate. The resulting mixture was stirred and the pH adjusted to about 8 by adding 10 M NaOH. The uncomplexed indium was removed by filtering the solution through a 0.2 μm Acrodisc filter.

The resulting mixture was analyzed by reversed phase HPLC with isocratic elution using 8% v/v solvent B and 92% v/v solvent A and compared with a sample of (p-aminobenzyl)EDTA. A single peak with a retention time greater than that of (p-aminobenzyl)EDTA indicated that all of the (p-aminobenzyl)EDTA was complexed with indium. The concentration of the indium complex was measured spectophotometrically as described above and used to prepare DBX.

B. Synthesis of (p-Nitrobenzyl)EDTA

(S)-p-nitrobenzyl EDTA was prepared and converted to (S)-4-isothiocyanatobenzyl EDTA ("ITCBE") as described in Meares et al., U.S. Patent No. 4,622,420, and Meares, Anal. Biochem. 142:68-75 (1984). The lyophilized ITCBE was resuspended in 0.3 M Ultrex HCL to a final concentration of approximately 50 mM ("Stock ITCBE solution"). This solution was stored at -70°C.

C. Synthesis of DBX

Indium p-aminobenzyl-EDTA (In-ABE) (50 mM, 250 μl) was mixed with p-aminobenzyl-DTPA (137 mM, 100 μl) and the solution diluted with 0.5M $NaHCO_3$, pH 9 (350 μl). Excess N,N-disuccinimidyl carbonate was added and the reaction vortexed periodically. Three products were evident in the HPLC analysis (3.2, 3.7, and 4.2 min) in a 1:2:1 ratio, consistent with urea formation. The reaction mixture was diluted to 50 mL with water and applied to an A-25 column (12 mL). The column was eluted with a gradient (200 mL) of 100 mM to 1M ammonium formate, pH 8. Fractions were combined based on HPLC analysis and the fraction containing the 3.7 min peak analyzed. HPLC analysis showed that this compound bound both indium (4.2 min) and yttrium (4.3 and 4.4 min). UV analysis showed the compound had an absorbance maximum at 260 nm. Incorporation of ^{111}Indium (as explained above) was >95% and CHA-255 binding of the ^{111}Indium (III) was 94%. The ^{13}C NMR spectrum contained a signal at δ 156.5, consistent with a urea carbon, as well as signals corresponding to Indium benzyl EDTA and benzyl DTPA. The proton NMR spectrum was consistent with the assigned structure.

NMR (^{13}C, D_2O, pH=4.5) δ: 177.2, 176.7, 176.6, 176.3, 171.2, 171.1, 155.8, 138.3, 138.0, 132.2, 131.1, 130.7 (CH), 121.7 (CH), 63.4 (CH), 62.0 (CH), 60.6 (CH_2), 58.7 (CH_2), 58.2 (CH_2), 57.7 (CH_2), 55.8 (CH_2), 55.0 (CH_2), 54.3 (CH_2), 54.0 (CH_2), 52.4 (CH_2), 49.5 (CH_2), 33.0 (CH_2), 32.5 (CH_2); (proton NMR, D_2O, pH=4.5) δ: 7.25 (d, 2H, $\underline{J}$ = 7.8 Hz), 7.23 (d, 2H, $\underline{J}$ = 7.8 Hz), 7.15 (d, 2H, $\underline{J}$ = 7.8 Hz), 7.09 (d, 2H, $\underline{J}$ = 7.8 Hz), 2.20 - 3.80 (32H).

EXAMPLE VII

Synthesis of ^{111}In-DBX

Indium chloride (In-111) was diluted to 2.5 mCi/ml with 0.05 M HCl. Buffered indium chloride (BIC)

was prepared by mixing 60 μl of the diluted indium chloride and 40 μl of 0.22 M glycine (pH 8.2).

The indium-111 labeled DBX was prepared by adding 160 μl (400 μCi) of BIC at 2.5 mCi/ml to 160 μl of DBX at 2,000 pmol/ml. The mixture was incubated for 30 minutes at room temperature. An equivalent volume (320 μl) of a solution containing 150 mM ascorbate, 50 mM tris, 0.1 mM DTPA (pH 7.4) was added to quench the reaction. Samples (1 μl) of the reaction were spotted on the origin of silica gel plates and allowed to dry completely. Buffered indium chloride radioisotope (0.5 μl) was also spotted on the plate as a control. The plate was placed in a thin layer chromatography (TLC) chamber that had been equilibrated with 10% ammonium acetate (aq):methanol (1:1) solution. Following chromatography, the plates were dried and cut from the bottom to slightly above the origin and from the top to the solvent front. The strips were transferred to gamma counting tubes and the amount of radioactivity of each strip was determined. Counts in the solvent front represent ^{111}Indium-labeled DBX. Counts remaining at or near the origin represent BIC. Routinely, 98% of the counts are incorporated into the DBX.

EXAMPLE VIII

In Vivo Biodistribution Studies

Tumors were established by subcutaneously injecting 1 x 10^6 BROtMDR1 cells from tissue culture into nude mice. BROtMDR1 cells were obtained from Dr. Piet Borst (Netherlands Cancer Institute, Amsterdam). The BROtMDR1 cell line was developed from the human melanoma cell line BRO following transfection of the human liver mdrl gene. When the tumors had reached 0.6-0.8 grams in size, 20 μg of Fab'$_3$ trifunctional compound, also referred to herein as $(LS2H)_2$xCHA, was injected into the tail vein

of the mice. Twenty-four hours later, 10 μCi of ^{111}Indium-labeled DBX was injected into the tail vein of each mouse.

At 4, 24, 48 and 120 hours after injection of the radiolabeled hapten, the animals were sacrificed and the biodistribution of the radioactivity was determined. Tumors, visceral organs, blood, bone, skin, muscle, urine and feces were weighed and counted in a gamma counter for activity. The amount of activity remaining in the tail was subtracted from the injected dose to calculate the percentage of the injected dose per gram of tissue.

The results of the biodistribution study are summarized in Figure 4. The trivalent antibody $(LS2H)_2$xCHA targeted the human drug resistant tumor xenograft and bound minimally to all the normal tissues that were evaluated. By 25 hours 13% dose/gram was localized in the tumor and remained at the tumor by 48 hours (14% dose/gram), while all the normal tissues, with the exception of kidney, had levels under 5% dose/gram. The kidney contained 7% dose/gram, which reflects the rapid clearance of the labeled DBX. The rapid clearance of DBX is also shown in the high counts in urine. Thus, the results of these studies indicate that the trifunctional compounds of the present invention have diagnostic and therapeutic utility to detect and treat multi-drug resistant tumors, including colon, renal, breast and ovarian carcinomas.

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It will be apparent to those skilled in the art that changes and modifications will be possible without departing from the spirit and scope of the invention. It is tended that the following claims be interpreted to embrace all such changes and modifications.

We claim:

1. A trifunctional compound having the formula:

$$\begin{array}{ccccc} X_1 & \text{———} & L & \text{———} & X_3 \\ & & | & & \\ & & X_2 & & \end{array}$$

wherein L is a cross-linking agent;

wherein X_1, X_2 and X_3 are Fab′-like moieties; and,

wherein at least one of said Fab′-like moieties has specific reactivity with a P-glycoprotein associated with a multi-drug resistant cell.

2. The trifunctional compound of claim 1, wherein said P-glycoprotein has an apparent molecular weight in the range of about 170-180 Kd.

3. The trifunctional compound of claim 1, wherein at least one of said Fab′-like moieties is derived from monoclonal antibody LS2H241.

4. The trifunctional compound of claim 1, wherein said trifunctional compound is a bi-specific compound.

5. The trifunctional compound of claim 4, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity with a diagnostic agent, and the third Fab′-like moiety has specific reactivity with said P-glycoprotein or said diagnostic agent.

6. The trifunctional compound of claim 5, wherein said diagnostic agent is a complex of a chelating agent and an imaging agent.

7. The trifunctional compound of claim 6, wherein said complex is a physiologically compatible chelate complex.

8. The trifunctional compound of claim 6, wherein said imaging agent is a radionuclide or a paramagnetic metal ion.

9. The trifunctional compound of claim 8, wherein said radionuclide is selected from the group consisting of ^{113m}In, ^{67}Ga, ^{99m}Tc, ^{51}Cr, ^{197}Hg, ^{203}Hg, ^{169}Yb, ^{85}Sr, ^{87}Sr and ^{111}In.

10. The trifunctional compound of claim 8, wherein said paramagnetic metal ion is selected from the group consisting of lanthanide elements of atomic number 57-70 and transition metals of atomic number 21-29, 42 or 44.

11. The trifunctional compound of claim 7, wherein said chelating agent is DBX.

12. The trifunctional compound of claim 4, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity with a therapeutic agent and the third Fab′-like moiety has specific reactivity with said P-glycoprotein or said therapeutic agent.

13. The trifunctional compound of claim 12, wherein said therapeutic agent is a cytotoxic agent or a complex of a chelating agent and a radionuclide.

14. The trifunctional compound of claim 13, wherein said cytotoxic agent is selected from the group consisting of doxorubicin, daunorubicin, mitomycin C, etoposide, vinblastine, vincristine, colchicine, dihydroxyanthracenedione, mitoxanthrone, mithramycin, actinomycin D, puromycin, gramicidin D, ethidium bromide, taxol, tenoposide, cytochalasin B, 1-dehydrotoastosterone.

15. The trifunctional compound of claim 13, wherein said radionuclide is selected from the group consisting of ^{67}Cu, ^{186}Rh, ^{188}Rh, ^{189}Rh, ^{153}Sm, ^{111}In (AÜger) and ^{90}Y.

16. The trifunctional compound of claim 13, wherein said chelating agent is DBX.

17. The trifunctional compound of claim 1, wherein said trifunctional compound is tri-specific.

18. The trifunctional compound of claim 17, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity with a diagnostic agent and the third Fab′-like moiety has specific reactivity with a therapeutic agent.

19. The trifunctional compound of claim 18, wherein said diagnostic agent is a complex of a chelating agent and an imaging agent.

20. The trifunctional compound of claim 19, wherein said chelating agent is DBX.

21. The trifunctional compound of claim 10, wherein said imaging agent is a radionuclide or a paramagnetic metal ion.

22. The trifunctional compound of claim 18, wherein said therapeutic agent is a cytotoxic agent or a complex of a chelating agent and an imaging agent.

23. The trifunctional compound of claim 22, wherein said complex is a physiologically compatible chelate complex.

24. The trifunctional compound of claim 23, wherein said chelating agent is DBX and said imaging agent is a radionuclide or a paramagnetic metal ion.

25. The trifunctional compound of claim 1, wherein said cross-linking agent is a tris-maleimide.

26. The trifunctional compound of claim 25, wherein said tris-maleimide is N,N′-bis(3-maleimidopropionyl)-2-hydroxy-1,3-propanediamine.

27. A composition comprising the trifunctional compound of claim 1 and a pharmaceutically acceptable carrier.

28. The composition of claim 27, wherein at least one of said Fab′-like moieties is derived from monoclonal antibody LS2H241.

29. The composition of claim 28, wherein said trifunctional compound is bi-specific.

30. The composition of claim 29, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity for a diagnostic agent and the third Fab′-like moiety has specific reactivity with said P-glycoprotein or said diagnostic agent.

31. The composition of claim 29, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity with a therapeutic agent and the third Fab′-like moiety has specific reactivity with said P-glycoprotein or said therapeutic agent.

32. The composition of claim 27, wherein said trifunctional compound is tri-specific.

33. The trifunctional compound of claim 32, wherein one Fab′-like moiety has specific reactivity with said P-glycoprotein, a second Fab′-like moiety has specific reactivity with a diagnostic agent and the third Fab′-like moiety has specific reactivity with a therapeutic agent.

34. A method for inhibiting the activity of a P-glycoprotein associated with a multi-drug resistant cell, said method comprising contacting said multi-drug resistant cell with a trifunctional compound of claim 1.

35. The method of claim 34, wherein said compound has one Fab′-like moiety having specific reactivity with a first P-glycoprotein binding site and a second Fab′-like moiety having specific reactivity with a second P-glycoprotein binding site.

36. The method of claim 34, further comprising the step of contacting said multi-drug resistant cell with a chemosensitizer.

37. The method of claim 34, further comprising the step of contacting said multi-drug resistant cell with a MDR drug.

38. The method of claim 34, wherein contacting said multi-drug resistant cell with a trifunctional compound of claim 1 is accomplished by administering said trifunctional compound to a mammal having a disorder associated with said multi-drug resistant cell.

39. A method for detecting a multi-drug resistant cell, comprising:

(a) contacting a sample containing or suspected of containing said multi-drug resistant cell with a detectable trifunctional compound of claim 1, said trifunctional compound having at least one Fab′-like moiety specific for a diagnostic agent; and

(b) detecting the binding of said trifunctional compound to the sample, wherein binding indicates the presence of said multi-drug resistant cell in said sample.

40. A method for detecting tumors expressing P-glycoprotein in a mammal, comprising:

(a) administering a trifunctional compound of claim 1 to said mammal, wherein at least one of said Fab′-like moieties has specific reactivity with a diagnostic agent;

(b) administering a diagnostically effective amount of said diagnostic agent to said mammal; and

(c) detecting the binding of said trifunctional compound to said mammal, wherein binding of said compound to said mammal indicates the presence of said tumor in said mammal.

41. The method of claim 40, wherein at least one of said Fab′-like moieties is derived from monoclonal antibody LS2H241.

42. The method of claim 40, wherein said diagnostic agent is a complex of a chelating agent and an imaging agent.

43. The method of claim 40, wherein said trifunctional compound and said diagnostic agent are administered simultaneously.

44. The method of claim 40, wherein said trifunctional compound and said diagnostic agent are administered sequentially.

45. A method for treating a mammal having a disorder associated with multi-drug resistance, said method comprising:

(a) administering a therapeutically effective amount of a trifunctional compound of claim 1 to said mammal, wherein one of said Fab′-like moieties has specific reactivity with a therapeutic agent; and

(b) administering a therapeutically effective amount of said therapeutic agent to said mammal.

46. The method of claim 45, further comprising the step of administering a MDR drug to said mammal.

47. The method of claim 45, wherein at least one of said Fab′-like moieties is derived from monoclonal antibody LS2H241.

48. The method of claim 45, wherein said therapeutic agent is a cytotoxic agent or a complex of a chelating agent and a radionuclide.

49. The method of claim 45, wherein said trifunctional compound and said therapeutic agent are administered simultaneously.

50. The method of claim 45, wherein said trifunctional compound and said therapeutic agent are administered sequentially.

51. A method for diagnosing and treating a mammal having or suspected of having a disorder associated with multi-drug resistance, said method comprising:

(a) administering a trifunctional compound of claim 1 to said mammal, wherein a second Fab′-like moiety has specific reactivity with a diagnostic agent and the third Fab′-like moiety has specific reactivity with a therapeutic agent;

(b) administering a diagnostically effective amount of said diagnostic agent;

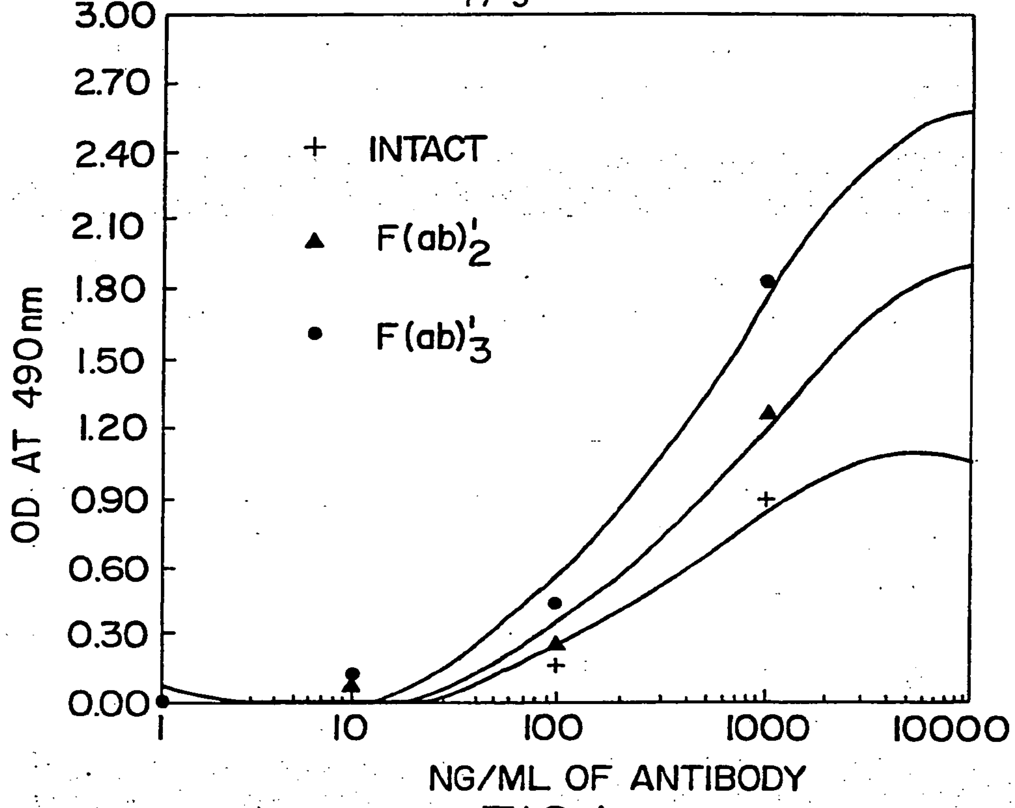
(c) detecting the binding of said trifunctional compound to said mammal, wherein binding of said compound to said mammal is diagnostic of said disorder; and

(d) if warranted, administering a therapeutically effective amount of said therapeutic agent to said mammal.

52. The method of claim 51, wherein said diagnostic agent is a chelating agent complexed to an imaging agent.

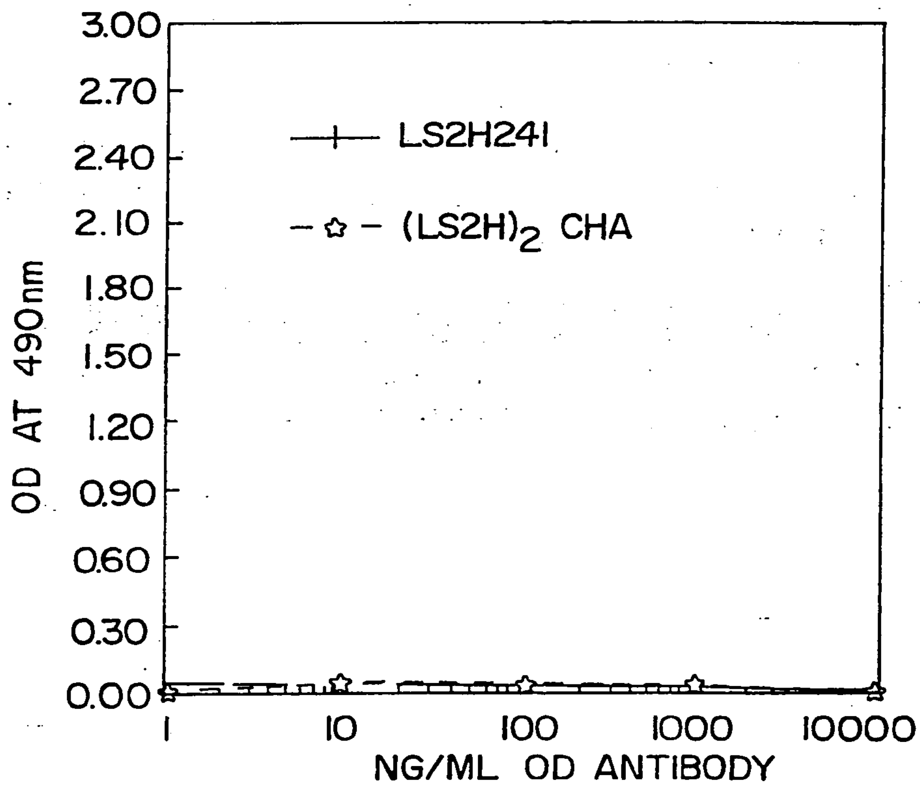
53. The method of claim 51, wherein said therapeutic agent is a cytotoxic agent or a complex of a chelating agent and a radionuclide.

54. A kit comprising the trifunctional compound of claim 1 and an ancillary reagent.



3.00
2.70
2.40
2.10
1.80
1.50
1.20
0.90
0.60
0.30
0.00
OD AT 490nm
+ INTACT
▲ F(ab)'2
● F(ab)'3
1
10
100
1000
10000
NG/ML OF ANTIBODY

FIG.1



3.00
2.70
2.40
2.10
1.80
1.50
1.20
0.90
0.60
0.30
0.00
OD AT 490nm
LS2H241
(LS2H)2 CHA
1
10
100
1000
10000
NG/ML OD ANTIBODY

FIG.2

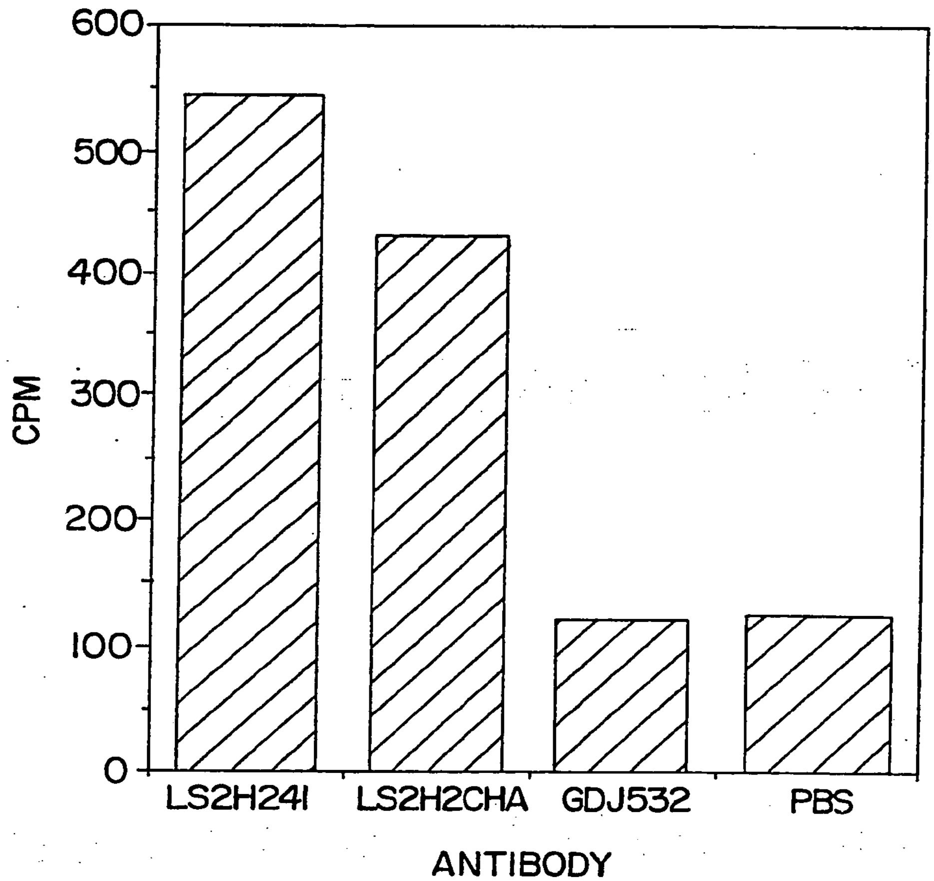

600
500
400
300
200
100
0
CPM
LS2H241
LS2H2CHA
GDJ532
PBS
ANTIBODY

FIG.3

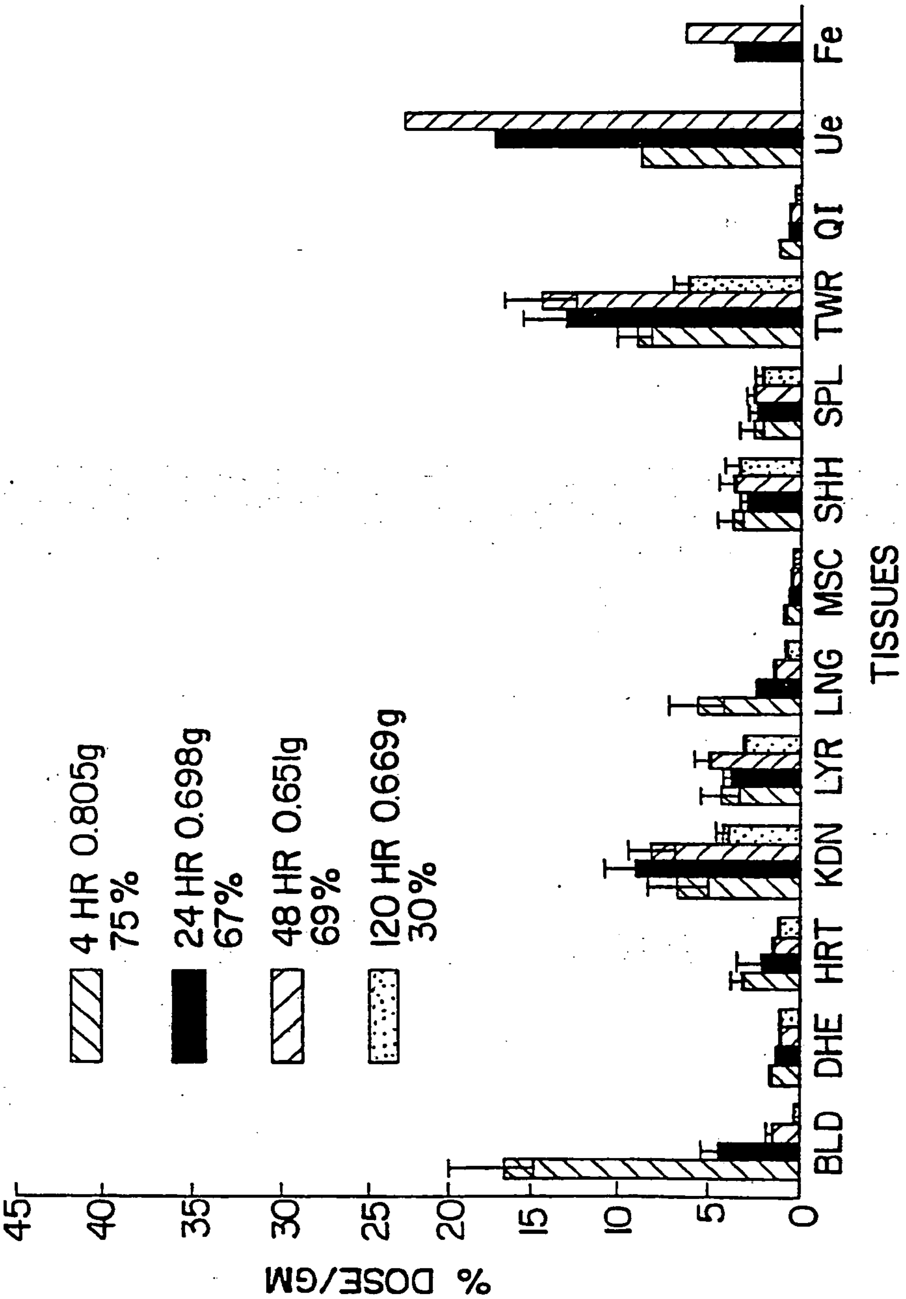

4 HR 0.805g 75%
24 HR 0.698g 67%
48 HR 0.651g 69%
120 HR 0.669g 30%
% DOSE/GM
0 5 10 15 20 25 30 35 40 45
BLD DHE HRT KDN LYR LNG MSC SHH SPL TWR QI Ue Fe
TISSUES

FIG.4

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/06043

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K15/00; A61K39/395; A61K49/00; A61K43/00
A61K47/48; A61K45/06; G01N33/577; G01N33/563

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; A61K ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
P,Y	EP,A,0 453 082 (HYBRITECH INCORPORATED) 23 October 1991 cited in the application see the whole document ---	1-39,54
Y	INTERNATIONAL JOURNAL OF CANCER vol. 44, no. 4, 15 October 1989, GENEVA, CH pages 738 - 743 J. VAN DIJK ET AL. 'BISPECIFIC ANTIBODIES REACTIVE WITH THE MULTIDRUG-RESISTANCE-RELATED GLYCOPROTEIN AND CD3 INDUCE LYSIS OF MULTIDRUG-RESISTANT TUMOR CELLS.' cited in the application see the whole document ---	1-39,54

-/--

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

1

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 OCTOBER 1992	02. 11. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	RYCKEBOSCH A.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8 601 407 (HYBRITECH INCORPORATED) 13 March 1986 cited in the application see page 7, line 16 - page 9, line 19; claims 1-10; examples 1,2 ---	1-39,54
A	CANCER RESEARCH vol. 49, no. 12, 15 June 1989, PHILADELPHIA, PA, US pages 3209 - 3214 M. B. MEYERS ET AL. 'CHARACTERIZATION OF MONOCLONAL ANTIBODIES RECOGNIZING A Mr 180,000 P-GLYCOPROTEIN: DIFFERENTIAL EXPRESSION OF THE Mr 180,000 AND Mr 170,000 P-GLYCOPROTEINS IN MULTIDRUG-RESISTANT HUMAN TUMOR CELLS.' cited in the application see the whole document -----	1-39,54

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 92/06043

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 40-53
because they relate to subject matter not required to be searched by this Authority, namely:
see PCT-Rule 39.1(iv)
Remark: Although claims (34,35)**,38 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.